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Ribozyme-mediated inhibition of a Philadelphia chromosome–positive acute lymphoblastic leukemia cell line expressing the p190 bcr-abl oncogene

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ABSTRACT

The *bcr-abl* oncogene is the molecular counterpart of the Philadelphia chromosome (Ph), which is detected in >95% of patients with chronic myelogenous leukemia (CML) and 20–30% of adults with acute lymphoblastic leukemia (ALL). Leukemic cells from patients with CML express the p210 form of the *bcr-abl* oncogene, whereas in adult Ph$^+$ ALL approximately 50% of cases express the p190 form of the *bcr-abl* oncogene, and the other 50% express the same p210 gene as is found in CML. In this study, we have designed hairpin ribozymes (RZs) specific for the p190 form of the *bcr-abl* oncogene to inhibit the growth of a p190 Ph$^+$ ALL cell line, Sup-B15. The RZs cleave p190 RNA substrate in a cell-free *in vitro* assay. In the presence of the liposome, DMRIE-C, the RZs are protected from serum mediated catalysis *in vitro*. Anti-p190 RZs transfected with DMRIE-C as the vector into K562 cells, which express the p210 *bcr-abl* oncogene, are stable intracellularly for up to 96 hours. Up to 33% of the DMRIE-C and RZ mixtures are taken up by Sup-B15 cells cultured in suspension. Expression of the p190 *bcr-abl* protein product is specifically inhibited as demonstrated by Western blot analysis. Cell growth of the Sup-B15 cells is completely inhibited by anti-p190 RZs over four days in culture. Anti-p210 RZs have no significant effect on *bcr-abl* protein expression or cell growth by Sup-B15 cells. RZs may have a role in purging stem cell populations collected from patients with Ph$^+$ ALL in the context of autologous bone marrow transplantation.

KEY WORDS

Ribozymes • Philadelphia chromosome • Acute lymphoblastic leukemia • p190 *bcr-abl*

INTRODUCTION

The Philadelphia chromosome (Ph) is detected in 20–30% of adult patients and in 2–6% of pediatric patients with acute lymphoblastic leukemia (ALL). In Ph$^+$ ALL, some patients express the typical p210 *bcr-abl* oncogene found in chronic myelogenous leukemia (CML), whereas other patients express a novel *bcr-abl* fusion gene that is transcribed to a smaller mRNA of 7.0–7.5 kbase size, and is translated into a smaller p190 protein that also has tyrosine kinase activity [1–3].

For both adult and pediatric patients with Ph$^+$ ALL, the long-term disease-free survival rate is extremely low without an allogeneic bone marrow transplant (BMT) [4–8]. Only about 30% of eligible patients will have a matched sibling available, and about 30% of the remaining patients may successfully find a matched unrelated donor through the National Marrow Donor Registry. Autologous BMT has been performed for Ph$^+$ ALL using a variety of purging techniques, usually some combination of monoclonal antibodies directed against common acute lymphoblastic leukemia antigens (CALLA) [9–11]. Most marrows harvested in clinical complete remission will have detectable residual disease by polymerase chain reaction (PCR) as reported by Gehly et al. [12]. It would be valuable to target specifically and to purge these residual *bcr-abl* positive cells from stem cell collections. One means of purging residual leukemia cells is to utilize antisense oligonucleotides (AS) [13,14]. Many investigators have demonstrated the efficacy of either anti-*bcr-abl* AS [15–22] or anti-c-*myb* AS [23,24] in inhibit-

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ing the growth of CML cells *in vitro* and *in vivo*. There is some uncertainty about the mechanism of action and the specificity of AS in these experiments [25–27].

Other investigators have used ribozymes (RZ) to purge specific target cells. RZ are a class of RNA molecules that can cleave other RNA sequences enzymatically [28–39]. RZs have the potential specifically to inhibit the expression of a variety of genes and to represent a novel therapeutic approach in controlling viral infections and oncogenesis [40]. RZs have been used to inhibit the expression of a variety of viral genomes, growth factors, and oncogenes [41–46].

We and other investigators have used RZs specifically designed to bind to the p210 *bcr-abl* oncogene to inhibit the growth of Ph^+ CML cell lines, as well as freshly isolated bone marrow cells from patients with CML [47–55]. In this study, we designed hairpin RZs specific for the p190 *bcr-abl* oncogene to inhibit expression of this oncogene in a p190 Ph^+ cell line. The hairpin design was chosen over a hammerhead RZ because a hairpin RZ can cleave at a site only one base to the 5′ side of the *bcr-abl* fusion site, whereas the closest target for a hammerhead RZ is located five or six bases to the 3′ side of the fusion site. The potential for cleavage of normal *abl* mRNA would therefore be greater with a hammerhead RZ compared with a hairpin RZ. We have used an efficient liposome vector, DMRIE-C, to transfect the RZs into the target cells. The uptake, stability, efficacy, and specificity of the RZs were studied in this system.

Figure 1. Sequences of hairpin ribozyme A
(A) Sequence of hairpin ribozyme A (HpA) and its target ALL bcr-abl mRNA. (B) Sequence of hairpin ribozyme A with T7 transcription terminator (HpA-Tm) and its target ALL bcr-abl mRNA.

MATERIALS AND METHODS

Synthesis of anti–ALL-*bcr-abl* RZ

A hairpin RZ to cleave ALL *bcr-abl* mRNA (HpA) (Fig. 1A) was synthesized in the City of Hope DNA Synthesis Core Facility. Two oligos, which together make up the RZs, overlap 13 bases at their 3′ end. A second set of shorter oligomers complimentary to the 5′ and the 3′ outer ends of these paired, overlapping oligos were used to amplify this product. From this PCR fill-in reaction was created a double-stranded template DNA containing a T7 RNA promoter from which RZ RNA could be transcribed. A T7 termination sequence, which was based on previous work, was also included on the template DNA (HpA-Tm) (Fig. 1B), thus demonstrating that this addition confers increased intracellular stability [56,57]. Milligram quantities of each RZ to be used in all experimental assays were made using the Ampliscribe T7 RNA transcription kit (Epicentre Technologies, Madision, WI). As a control for the anti–ALL-*bcr-abl* RZs, we used a hammerhead RZ directed against the splice 1 p210 *bcr-abl* gene (Spl1RZ), which we described previously [47].

Preparation of ALL *bcr-abl* RNA substrate

At the City of Hope Medical Center, the ALL substrate RNA was isolated from a bone marrow specimen taken from a patient with p190 *bcr-abl* positive ALL. The patient had given informed consent for this IRB-approved protocol. The cDNA generated by RT-PCR was subcloned into pBluescript II KS ± (Stratagene, La Jolla, CA) by the T/A cloning technique for PCR products and was confirmed by DNA sequencing. RNA was transcribed using T7 RNA polymerase and ^{32}P-UTP by MAXIscript kit (Ambion, Austin, TX).

Cell-free assay of RZ cleavage of ALL *bcr-abl* mRNA

The cell-free *in vitro* ribozyme cleavage assay was done as previously described [47] with one modification; instead of snap-cooling the heated RZ/substrate complex on ice, the heated complex was placed at 37°C for 10 minutes before adding the Mg^{2+} to 10 mM final concentration.

In vitro stability of RZ/liposome complexes

Five micrograms of RZ RNA was mixed with the liposome vector DMRIE-C (1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide formulated with cholesterol at a 1:1 M/M ratio in water; Gibco/BRL, Gaithersburg, MD) in varying ratios. After 30 minutes at room temperature with occassional mixing, an equal volume (15 μL) of different media were added: a) 1×phosphate-buffered saline (PBS), b) RPMI 1640 plus 10% fetal calf serum (FCS), c) and d) RPMI 1640 plus 10% FCS with either 2 or 4 g of DMRIE-C. These mixtures were incubated overnight at 37°C. The RNA was then extracted by RNAzol B solution (Cinna-Biotex, Houston, TX). The results were analyzed on a 6%/7M polyacrylamide gel (PAGE) and stained with ethidium bromide.

RZ uptake in Sup-B15 and K562 cells

Ten microgams of cold HpA-Tm was mixed with 5×10^5 counts per minute (cpm) of P^{32}–labeled HpA-Tm in 600 µl. of opti-MEM I reduced serum medium (Gibco BRL, Gaithersburg, MD). DMRIE-C was thoroughly vortexed and added in 0, 2, 4, 8, 12 and 16 µg amounts to 50 µl. opti-MEM medium in separate wells of a 24-well plate at room temperature for 30 minutes. Sup-B15 cells (ALL Ph^1 cell line, from American Type Culture Collection) were added to the RNA/DMRIE-C at a concentration of 5×10^5/well. This new mixture was allowed to incubate overnight at 37°C, 5% CO_2. The cells were washed in cold 1×PBS three times then the RNA was extracted using RNAzol B. The RNA pellet was then dissolved into 10 µl. of 2×denaturing buffer. Each sample was counted by the Cerenkov method on a counter.

To determine the intracellular uptake of RZ mediated by liposome, K562 cells were also transfected by RZ/DMRIE-C complexes. K562 cells were used in these assays since they express the splice 1 p210 *bcr-abl* gene product, which cannot be bound or cleaved by the HpA RZ. The uptake and stability of the RZs in these cells could, therefore, be analyzed separately from these processes. At different time intervals, cells were washed three times and fixed in a 2% cacodylate buffered glutaraldehyde. After an additional 1% OsO_4 processing, the K562 cells were thin-sectioned and stained by Sato's lead solution and were observed using a Philips CM 10 Electron microscope.

Intracellular stability of RZ

Five micrograms of RZ containing 2.5×10^5 cpm of P^{32}-labeled RZ RNA was mixed with 4 µg of DMRIE-C in opti-MEM medium. After 30 minutes, 5×10^5 K562 cells were added to this complex so that the final volume was 500 µL. The cells were placed in a 37°C, 5% CO_2 humidified incubator overnight. The next morning the cells were washed twice in 10 mL of sterile 1×PBS and then the cells were resuspended in 4 mL of RPMI 1640 plus 10% FCS. One-milliliter volumes of the resuspended cells were placed in the appropriate wells of a 24 well plate. At time intervals of 24, 48, 72, and 96 hours, the cells were pelleted and washed three times with ice-cold 1×PBS. After the final wash, the cell pellet was loosened and the RNA was extracted. Each sample was counted by the Cerenkov method in a counter, analyzed on a 6%/7M PAGE, and subjected to an autoradiography.

RZ transfection of Sup-B15 cells and cell proliferation assay

The Sup-B15 cell line was maintained in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FCS in our laboratory. During transfection, the Sup-B15 cells were washed 2 times with opti-MEM medium and incubated with the same medium at a cell concentration of 7×10^5 per well. The cells were grown in duplicate in a 24-well plate for 1 hour before transfection. Next, 20 µg of either control RZs or specific hairpin RZs were mixed with 8 µg DMRIE-C in opti-MEM medium, and complexes were allowed to form. After 30 minutes, these complexes were added to the duplicate wells and incubated for 12 hours at 37°C. At 12 hours, IMDM with 20% FCS was added to each well to yield a final concentration of 4% FCS in opti-MEM medium. A second dose of 10 µg RZs and 4 µg DMRIE-C mixture was added 24 hours after the first dose. Sup-B15 cell counts and viability (trypan blue exclusion) were determined daily until the fourth day. The cell proliferation assay was repeated three times. The final growth curve shows the means of duplicate samples at each time interval.

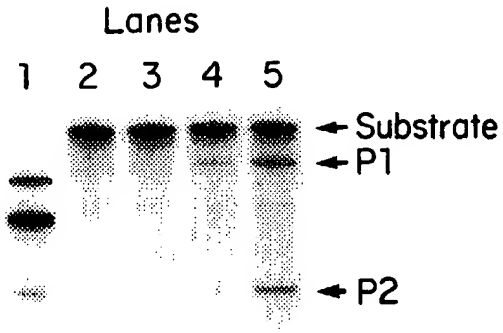

Figure 2. Cell-free cleavage of ALL bcr-abl mRNA substrate
Lane 1, size markers; lane 2, ALL substrate incubated with anti–p210 bcr-abl ribozyme; lane 3, ALL substrate alone; lane 4, ALL substrate incubated with HpA ribozyme; lane 5, ALL substrate incubated with HpA-Tm ribozyme.

Western blotting assay of $p190^{bcr\text{-}abl}$ protein

Sup-B15 cells transfected with RZs for 72 hours were extracted for cellular proteins as described previously [58] with some modification. In brief, cells were incubated overnight with the RZs in serum-free medium, then cultured for 72 hours in FCS-containing medium before the protein was extracted. The protein samples were separated by SDS-PAGE and transferred electrophoretically. The nitrocellulose membrane was immunoblotted with anti-bcr and anti-abl monoclonal antibodies (bcr-AB2, c-abl-AB3; Oncogene Science, Manhasset, NY). The signals were detected with ECL Western blotting reagents (Amersham, Arlington Heights, IL).

RESULTS

In vitro cell-free cleavage of substrate

Figure 2 shows the results of *in vitro* cell-free cleavage of p190 *bcr-abl* substrate by both the HpA and HpA-Tm RZs in the presence of Mg^{++}, at an approximate 1:1 ratio of substrate to RZ. Two products of the expected sizes are detected after overnight incubation. There was slightly more cleavage product seen with the HpA-Tm compared with the HpA RZ. As a control, a RZ designed to cleave splice 1 p210 *bcr-abl* mRNA (b3a2) was also incubated with the ALL substrate, but no cleavage was observed.

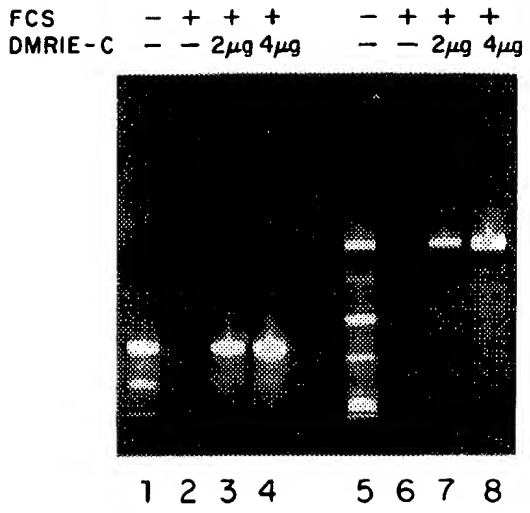

Figure 3. In vitro stability of ribozymes, HpA (lanes 1–4), and HpA-Tm (lanes 5–8)

Lanes 1 and 5, degradation of ribozymes in medium alone without FCS; lanes 2 and 6, complete degradation of ribozymes in 10% FCS; lanes 3 and 7, protection by 2 g of DMRIE-C; lanes 4 and 8, protection by 4 g of DMRIE-C.

In vitro stability of RZ

Figure 3 shows the effects of overnight incubation of the two RZs, HpA, and HpA-Tm in the presence of FCS, and the protective effects of DMRIE-C. Lanes 1 and 4 show the degradation patterns of the RZs in the absence of FCS. The RZs are completely degraded in the presence of 10% FCS (lanes 2 and 5), and DMRIE-C protects the RZs from this degradation (lanes 3 and 4 for HpA; lanes 7 and 8 for HpA-Tm).

RZ uptake by Sup-B15 and K562 cells

Figure 4 shows the results of RZ uptake by Sup-B15 cells when mixed with varying amounts of the DMRIE-C liposome. The ratio of 10 g of RZ to 8 g of DMRIE-C was the most effective, with approximately 22–33% (mean of 27.5%) of the RZ taken up in two repeated experiments. In analogous experiments, K562 took up 56% of the HpA RZ with an optimized ratio of 10 g of RZ to 6 g of DMRIE-C (data not shown). Less RZ was taken up at both lower and higher concentrations of the liposome.

Figures 5A–D are electron micrographs that show that the RZ/DMRIE-C complex was taken up by the K562 cells and located in the cytoplasm within endosomes after one hour of incubation. The complexes were not detected in the nucleus even after 24 hours of incubation.

Intracellular stability of RZ

P^{32}-labeled HpA and HpA-Tm RZs were mixed with cold RZs and transfected into K562 cells in the presence of DMRIE-C. At various time intervals, the RNA was extracted from the cells and analyzed by PAGE. Figure 6 shows that 68 and 72% of the intracellular HpA and Hpa-Tm RZs recovered at 24 hours were still recoverable after 96 hours of incubation, respectively. There was no significant difference between the amount of the two RZs recovered by 96 hours, although the kinetics of degradation of the two RZs were somewhat different. The HpA RZ appeared to be more stable during the first 48 hours of culture, and the HpA-Tm RZ seemed more stable during the last 72 hours.

Inhibition of p190 protein expression by Western Blot

Protein was isolated from Sup-B15 cells after incubation with various RZs, and detected by the Western Blot technique with monoclonal antibodies directed against both normal abl and normal bcr proteins. Figure 7 shows that expression of the p190 *bcr-abl* protein product is inhibited by HpA-Tm and HpA RZs, but not by spl1RZ which is directed against the p210 *bcr-abl* gene found in K562 cells. The relative densities of the p190 band in the control Sup-B15 cells compared with the cells incubated with HpA-Tm, HpA, and spl1RZ were 100, 14.8, 39.3, and 92.0%, respectively. There were no significant differences in the densities of internal control bands for these four samples.

Inhibition of cell growth

Sup-B15 cells were incubated for 4 days as described in the Methods section. As shown in Figure 8, cell growth was

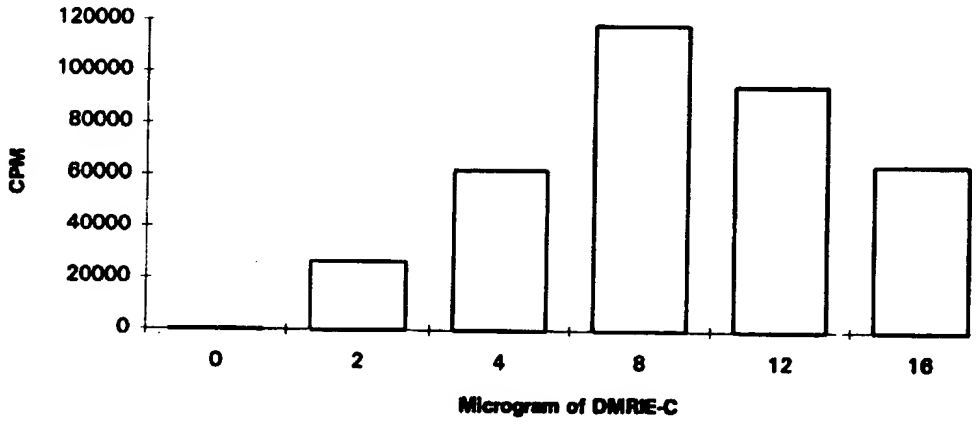

Figure 4. DMRIE-C–mediated uptake of HpA-Tm ribozyme by Sup-B15 cells

In this experiment, maximum uptake was 22% with 8 g DMRIE-C and 10 g "cold" ribozyme. Uptake of 33% was seen in a repeat experiment for a mean of 27.5%.

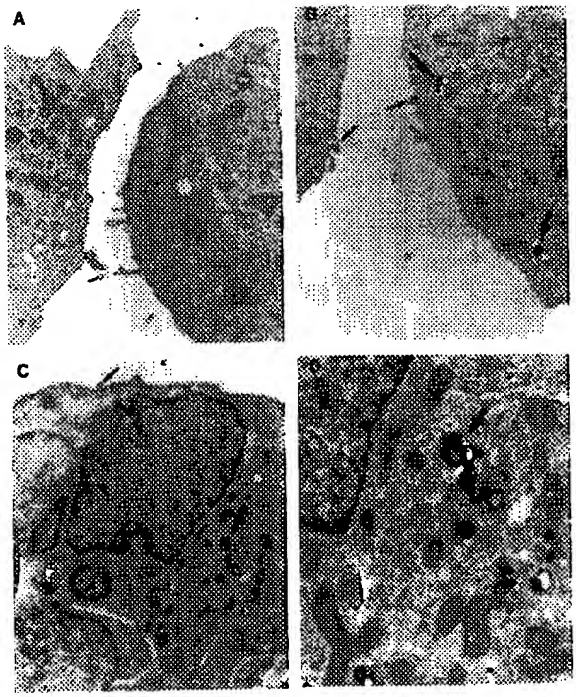

Figure 5. Electron micrographs of K562 cells transfected with HpA ribozyme/DMRIE-C complexes

(A) Control cells, ×22,250. (B) and (C) At 1 hour, complexes are visible on cell surfaces (small arrows) and in endosomes (large arrows), ×22,250. (D) At 1 hour, ×52,500.

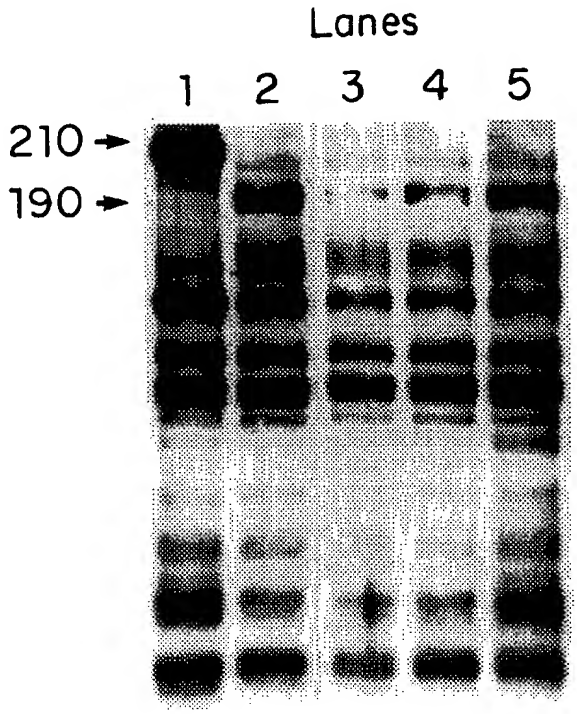

Figure 7. Western Blot analysis of inhibition of bcr-abl protein expression by Sup-B15 cells transfected with ribozymes/DMRIE-C complexes

Lane 1, K562 cells expressing p210 bcr-abl protein; lane 2, Sup-B15 cells expressing p190 bcr-abl protein (density of p190 band is 100%); lane 3, Sup-B15 cells transfected with HpA-Tm ribozyme (density of p190 band relative to lane 2 is 14.8%); lane 4, Sup-B15 cells transfected with HpA ribozyme (density of p190 band relative to lane 2 is 39.3%); lane 5, Sup-B15 cells transfected with anti–p210 bcr-abl ribozyme (density of p190 band relative to lane 2 is 92.0%).

inhibited significantly by both HpA and HpA-Tm RZs. The cell counts represent viable cells at each time interval. The percentage of dead cells (as determined by trypan blue dye exclusion) increased from 5% at the start of incubation to 10% after 4 days for the experimental groups displayed in Figure 8. Spl1RZ had no significant effect. Figure 8 shows the results of a representative experiment that was repeated three times with similar results.

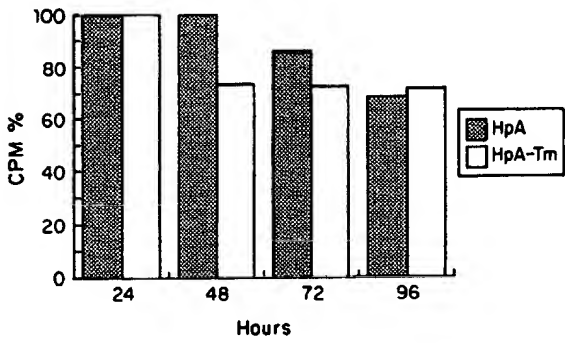

Figure 6. Intracellular stability of ribozymes transfected into K562 cells by DMRIE-C

Shaded bars, HpA; open bars, HpA-Tm. Five grams of ribozyme containing 2.5×10^5 cpm of P^{32}-labeled RZ RNA was mixed with 4 g of DMRIE-C in opti-MEM medium. Recovered radioactivity at 24 hours was normalized to 100%.

DISCUSSION

Ph^1 ALL in adult and pediatric patients is a high-risk form of ALL with poor long-term survival rates in the absence of an allogeneic BMT [6–8]. Autologous stem cell transplants have been attempted for patients who lack an allogeneic donor, with disappointing overall results because of high relapse rates [6,7]. Gene-marking studies in patients with neuroblastoma, acute myelogenous leukemia [59,60], and CML [61] have demonstrated that reinfused stem cells contribute to relapses after autologous transplantation. We have been investigating the feasibility of using ribozymes specific for the *bcr-abl* oncogene as a method to purge residual Ph^1 ALL cells from mobilized stem cells.

In this study, we have demonstrated that a hairpin RZ, designed to cleave this unique fusion RNA, can be delivered to target cells by an efficient transfection system and can inhibit gene expression and cell growth. An RZ with an added T7 termination sequence was tested because there are data to suggest that such a construct would be more stable intracellularly. In our experiments, there was no significant advantage demonstrated for this construct, per-

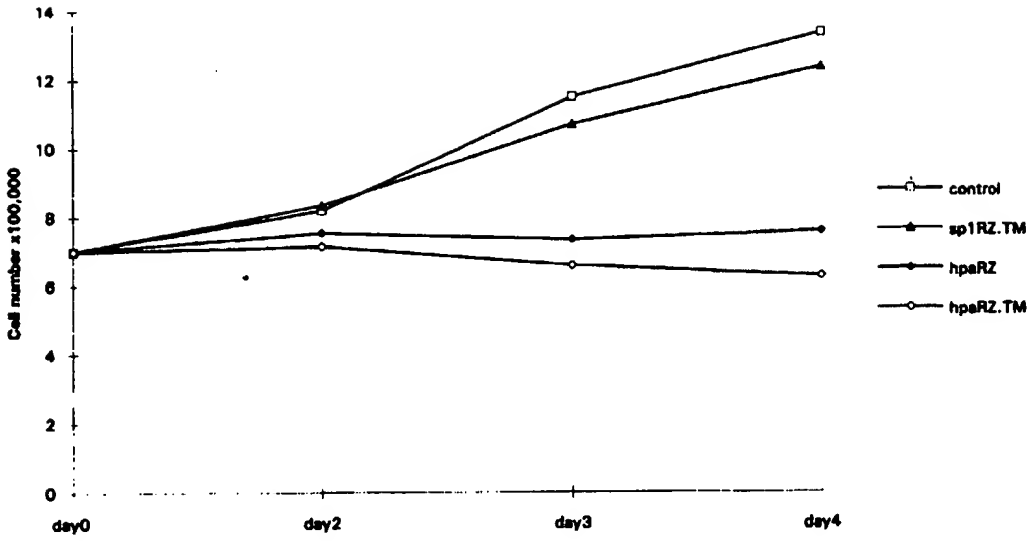

Figure 8. Inhibition of Sup-B15 cell growth by ribozymes

Open squares (□), control cells; closed triangles (▲), sp1RZ.TM = anti-p210 ribozyme linked to T7 transcription terminator; closed diamonds (◆), HpA ribozyme; open diamonds (◇), HpA-Tm ribozyme.

haps because of the increased stability conferred by the DMRIE-C liposome complexes.

The DMRIE-C vector has been shown to transfect suspension cells including K562 cells with a very high efficiency, using a β-galactosidase expression assay [62]. This liposome was more efficient than several other reagents in transfecting suspension cells based on a chloramphenical acetyl transferase assay [62]. In our system, DMRIE-C was able to stabilize the hairpin RZ when incubated together in serum. It also mediated efficient uptake of the RZ by target cells. RZ/DMRIE-C complexes were demonstrated to be taken up intracellularly in endosomes by EM. The intracellular RZ remained quite stable, with approximately 70% of the RZ present at 24 hours still recoverable at the end of the 96 hours.

The transfected RZ/DMRIE-C complexes were not only taken up by the Sup-B15 target cells, but they also were effective in inhibiting p190 *bcr-abl* gene expression, as demonstrated by the Western blot results. The p190 protein product was significantly reduced by the anti-p190 RZ but not by the anti-p210 RZ used as a control. In addition, the anti-p190 RZs were very effective in inhibiting cell growth of Sup-B15 cells, whereas the anti-p210 RZ had no significant effect. We are currently investigating the impact of the RZs on apoptosis of Sup-B15 cells.

These studies demonstrate that anti-p190 *bcr-abl* RZs can be efficiently delivered to target cells and inhibit gene expression and cell growth. This transfection system using DMRIE-C liposome is highly efficient, though it only allows for short-term expression of the RZ intracellularly. This approach may be an effective one for purging stem cell populations isolated from patients with Ph^1 ALL to be used in the context of autologous stem cell transplants. It is our intention to test the efficacy and safety of this approach in both a p190-*bcr-abl* transgenic mouse model and in a SCID mouse model of Ph^1 ALL, and then to apply it in a clinical setting.

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